DNA Microarray-Based Identification of Genes Controlled by Autoinducer 2-Stimulated Quorum Sensing in *Escherichia coli*

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Bacterial cell-to-cell communication facilitates coordinated expression of specific genes in a growth rate-II and cell density-dependent manner, a process known as quorum sensing. While the discovery of a diffusible *Escherichia coli* signaling pheromone, termed autoinducer 2 (AI-2), has been made along with several quorum sensing genes, the overall number and coordination of genes controlled by quorum sensing through the AI-2 signal has not been studied systematically. We investigated global changes in mRNA abundance elicited by the AI-2 signaling molecule through the use of a *luxS* mutant that was unable to synthesize AI-2. Remarkably, 242 genes, comprising ca. 5.6% of the *E. coli* genome, exhibited significant transcriptional changes (either induction or repression) in response to a 300-fold AI-2 signaling differential, with many of the identified genes displaying high induction levels (more than fivefold). Significant induction of *ygeV*, a putative σ[54]-dependent transcriptional activator, and *yhbH*, a σ[54] modulating protein, suggests σ[54] may be involved in *E. coli* quorum sensing.

Many bacteria have evolved the ability to condition culture medium by secreting low-molecular-weight signaling pheromones in association with growth phase to control expression of specific genes, a process termed quorum sensing (19). Physiological processes controlled by quorum sensing occur in diverse species of bacteria and include bioluminescence (17), antibiotic biosynthesis (4), pathogenicity (34), and plasmid conjugal transfer (18). While acyl-homoserine lactones (HSL) appear to be the predominant quorum signal (or autoinducer [AI]) used by host-associated gram-negative bacteria, discovery of a second signaling pathway in the marine bacterium *Vibrio harveyi* (6, 8, 41) revealed an alternate AI, termed AI-2, which regulates bioluminescence in conjunction with AI-1 (*N*-(3-hydroxybutanoyl)-L-homoserine lactone) (7).

Importantly, AI-2 (or AI-2-like) activity has been observed in virtually all strains of pathogenic and nonpathogenic *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (16, 40–42), requiring the *luxS* gene for synthesis (43). The physiological role of AI-2 in *E. coli* has not been clearly elucidated, but initial findings indicate that inhibition of chromosomal replication was subject to a quorum sensing mechanism (52). More recently, quorum sensing in *E. coli* has been implicated in regulating the expression and activity of SdiA, a LuxR-type transcriptional activator of the cell division genes *ftsQAZ*, through AI-2 (15, 39). In addition, extracellular factors which accumulate in enterohemorrhagic *E. coli* O157:H7 culture supernatants bind to the N-terminal region of SdiA for controlling the expression of virulence factors in a quorum-dependent fashion (25). Besides possible roles in cell division and pathogenesis, quorum sensing in *E. coli* was postulated to play a role

in stationary phase gene expression (23, 27, 39), perhaps in a bimodal fashion with the stationary phase sigma factor *rpoS* or with other yet-to-be-determined quorum signals.

Recently, the application of global identification methodologies (e.g., DNA microarrays) has resulted in identification of quorum-regulated processes as well as the characterization of quorum circuit architecture in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (16, 51). Therefore, a systematic investigation of native, quorum-mediated genes in *E. coli* was performed here to quantitatively analyze the global transcriptional pattern in response to the extracellular AI-2 signal molecule. To this end, DNA microarray analysis was utilized to quantify changes in transcription for every open reading frame (ORF) of *E. coli* strain W3110 in response to AI-2 signaling molecule.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* strains used in this study were W3110 (F⁻ λ⁻ IN(*rrnD-rrnE*) *rph-1*) (22), *E. coli* Genetic Stock Center, New Haven, Conn., and MDAI2, a *luxS*::Tc^r derivative of W3110 (16). *V. harveyi* strains BB152 (*luxL*::Tn5 AI-1⁻, AI-2⁺) and BB170 (*luxN*::Tn5 sensor 1⁻, sensor 2⁺) for determination of AI-2 activity (41) were kindly provided by B. L. Bassler. Plasmid pGFPuv-*ftsQ*2p for quantifying quorum-regulated *ftsQ*2p expression is described elsewhere (15). Luria-Bertani (LB) medium contained 5 g of yeast extract (Sigma Chemical Co.) per liter, 10 g of Bacto tryptone (Difco) per liter, and 10 g of NaCl per liter and was supplemented with 50 mM glucose. Autoinducer bioassay medium and LM medium (L-marine) are given in detail elsewhere (8, 37).

Growth conditions. Overnight *E. coli* MDAI2 cultures grown aerobically in LB broth plus supplemental glucose (50 mM) were subcultured into 200 ml of LB plus 50 mM glucose (1% [vol/vol] inoculum). Cultures were grown aerobically at 30°C to an optical density at 600 nm (OD$_{600}$) of 1.0 followed by centrifugation (2,500 × g at 4°C) and gentle resuspension in ~1 ml of fresh LB. Resuspended cells were split equally into two parallel flasks each containing 100 ml of conditioned medium (positive or negative for AI-2) plus 50 mM glucose (prepared as outlined below) such that the culture OD$_{600}$ was maintained at ~1.0. Aerobic growth ensued for 20 min, at which time 5-ml samples were collected for total RNA extraction.

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Report Documentation Page

1. REPORT DATE **2001**	2. REPORT TYPE	3. DATES COVERED **00-00-2001 to 00-00-2001**

4. TITLE AND SUBTITLE **DNA Microarray-Based Identification Of Genes Controlled ByAutoinducer 2-Stimulated Quorum Sensing In Escherichia coli**	5a. CONTRACT NUMBER
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Edgewood Research, Development, and Engineering Center,Aberdeen Proving Grounds,Aberdeen,MD,21001**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release; distribution unlimited**

13. SUPPLEMENTARY NOTES **Journal of Bacteriology, Sept. 2001, pages 5239-5247, Vol. 183, No. 18**

14. ABSTRACT

Bacterial cell-to-cell communication facilitates coordinated expression of specific genes in a growth rate-II and cell density-dependent manner, a process known as quorum sensing. While the discovery of a diffusible Escherichia coli signaling pheromone, termed autoinducer 2 (AI-2), has been made along with several quorum sensing genes, the overall number and coordination of genes controlled by quorum sensing through the AI-2 signal has not been studied systematically. We investigated global changes in mRNA abundance elicited by the AI-2 signaling molecule through the use of a luxS mutant that was unable to synthesize AI-2. Remarkably, 242 genes, comprising ca. 5.6% of the E. coli genome, exhibited significant transcriptional changes (either induction or repression) in response to a 300-fold AI-2 signaling differential, with many of the identified genes displaying high induction levels (more than fivefold). Significant induction of ygeV, a putative s54-dependent transcriptional activator, and yhbH, a s54 modulating protein, suggests s54 may be involved in E. coli quorum sensing.

15. SUBJECT TERMS					

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**9**	

Preparation of cell-free culture fluids and conditioned medium. W3110 (*luxS*$^+$) and MDAI2 (*luxS*) overnight cultures, grown aerobically at 30°C in LB plus 50 mM glucose, were used to inoculate 500 ml (1% inoculum) of fresh LB plus 50 mM glucose. Cultures were grown to an OD$_{600}$ of 3.0 (~8 h), and glucose analysis (YSI glucose analyzer model 2700) was used to confirm identical growth patterns in addition to growth rate calculations based on culture OD$_{600}$. Cell-free culture fluids or conditioned medium (CM) was prepared by centrifugation of 500-ml *E. coli* cultures for 10 min (10,000 × *g* at 4°C). Cleared supernatants were passed through 0.22-μm vacuum-driven Millipore filters and were stored at −20°C. Prior to use in AI-2 signaling experiments, CM was supplemented with 50 mM glucose and was assayed for AI-2 activity to confirm signaling conditions (positive or negative for AI-2). *V. harveyi* BB152 cell-free culture fluids were prepared analogously to obtain positive (+AI-2) control samples as reported previously (41).

AI activity assay. *E. coli* cell-free culture fluids were tested for the presence of AI-2 using the *V. harveyi* reporter strain BB170, which responds only to AI-2 (41). Luminescence assays were performed as outlined elsewhere (41), and luminescence was measured as a function of *V. harveyi* cell density by quantitating light production with a luminometer (EG & G Berthold). Data reported as fold activation were obtained by dividing the light produced by the reporter after addition of *E. coli* culture fluid by the light output of the reporter when growth medium alone was added (15).

Growth stimulation assays. Overnight cultures of W3110 and MDAI2 grown in LB were used to inoculate (1%, vol/vol) one of the following: LB plus 10% CM (+AI-2); LB plus 10% CM (+AI-2) plus 0.8% glucose; LB plus 10% CM (−AI-2); or LB plus 10% CM (−AI-2) plus 0.8% glucose. These experiments were performed in triplicate. OD$_{600}$ measurements were taken every 60 min over a 9-h period and used to calculate the specific growth rate for exponentially growing batch cultures. The specific growth rates were determined, with most accuracy for the first four data points.

RNA isolation and labeling. Harvested cells were centrifuged (5,000 × *g* at 4°C) and resuspended in lysozyme-containing buffer (5 μg μl^{-1} of lysozyme (Sigma) in 1× Tris-EDTA Buffer [Sigma], pH 8.0) at room temperature for 5 min. Total RNA was purified from 3 × 10^9 to 5 × 10^9 cells using a Qiagen RNeasy mini kit. RNA was eluted with diethyl pyrocarbonate (DEPC) water (Sigma) and quantified by measuring absorbance (A_{260}) with a spectrophotometer (Beckman DU 640). RNA samples (~75 to 80 μg) were concentrated to ~15 μl using a Microcon YM-30 filter (Millipore). Total purified RNA was labeled with either Cy3-dUTP or Cy5-dUTP as outline previously (49). Briefly, total RNA (~15 μl) was mixed with random hexamer primers (Amersham Pharmacia) and combined with 1X labeling mixture (first-strand buffer [Gibco-BRL], deoxynucleoside triphosphates (low TTP; Pharmacia), RNAsin (Promega), and DEPC water) and incubated at room temperature for 10 min. Labeling with Cy-3-dUTP (or Cy-5-dUTP) during a reverse transcriptase reaction using Superscript II (Gibco-BRL) was at 42°C for 1 h in the dark. After labeling, NaOH was added to the sample to hydrolyze RNA template and was incubated (65°C for 15 min) followed by neutralization with HCl and Tris (pH 7.6). Following Microcon MY-30 filter purification, control and experimental samples were combined, pulse centrifuged (<1 min), washed, and concentrated by filter centrifugation prior to direct hybridization to glass microarrays.

Microarray hybridization procedures. Glass DNA microarrays (University of Wisconsin Gene Expression Center), consisting of full-length PCR products (spotted one time) from all *E. coli* ORFs according to Blattner et al. (9), were used to quantify relative mRNA levels by parallel two-color hybridization according to protocols described elsewhere (49). The number of features on the slide, therefore, consisted of the 4,290 annotated ORFs plus ~200 control spots, including fragmented *E. coli* genomic DNA, nonspecific salmon sperm DNA, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), and six different yeast ORF PCR products. Slides were 36 spots by 36 spots by 4 panels, with each spot averaging 100 μ in diameter. Briefly, labeled probes were mixed with salmon sperm DNA, yeast tRNA, and PerfectHyb buffer (Sigma) prior to overnight hybridization to a DNA microarray (~12 h at 50°C). Arrays were washed with 0.2X SSC and 0.1% Sodium dodecyl sulfate (Sigma) for 2 min. Subsequent 2-min washes (three times) were with 0.2X SSC followed by dipping (C9. 10 times) in 0.05X SSC. Arrays were centrifuged to dry the surface and then scanned immediately using a GMS418 scanner (Genetic Microsystems) at 10-μm resolution and Arrayscan software (Genetic Microsystems). The resulting 16-bit TIFF images were analyzed using SCANALYZE software, publicly available at http://rana.stanford.edu/software/. RNA samples from experimental flasks (+AI-2 cultures) were first labeled with Cy5, and control RNA (−AI-2 culture) was labeled with Cy3. Reverse labeling of the RNA (experiment, Cy3; control, Cy5) was then performed on a second array and used to verify all transcriptional induction ratios. Analogously to procedures of LaRossa and colleagues (50), further vali-

TABLE 1. AI-2 activity of CM experienced by MDAI2 *luxS* cells

CM (+AI-2 or −AI-2)	Fold AI-2 activity[a]
CM stock solutions	
CM generated from W3110 *luxS*$^+$	1,520
CM generated from MDAI2 *luxS*	5
Experimental samples	
MDAI2 cells at an OD$_{600}$ of 1.0	7
MDAI2 cells (control, *t* = 0 min)	6
MDAI2 cells (exp, *t* = 0 min)	1,498
MDAI2 cells (control, *t* = 20 min)[b]	5
MDAI2 cells (exp, *t* = 20 min)[b]	1,514

[a] Fold AI-2 activity represents the induction of luminescence (RLU) in *V. harveyi* reporter strain BB170 (sensor 1$^-$, sensor 2$^+$) from *E. coli* cell-free culture samples in relation to luminescence (RLU) with fresh medium alone. Values are reported as the averages of three independent activity measurements.
[b] Samples used for RNA isolation and Cy3 or Cy5 labeling and subsequent hybridization to microarray. OD$_{600}$S of these cultures were almost identical (OD$_{600}$ = 1.2). Fold induction results from three replicate experiments agreed within 10%. exp, experimental.

dation of microarray data was made via independent measures of RNA transcript level by RNA dot blots as outlined previously (15).

Data selection and analysis. Microarray data whose intensities were reproducibly higher than that of the background level were selected for analysis to eliminate expression ratios that were extremely high or low due to undetectable signal in control or experimental samples (26). Induction ratios (Cy5 for experimental RNA relative to Cy3 for control RNA) as well as background intensities for each dye were independently distributed in approximately normal fashion. Induction ratios were obtained by dividing background-corrected signal intensities of experimental samples by background-corrected intensities of control samples. Intensities used to calculate induction ratios were normalized as a percentage of the total of intensities of all the spots (Cy5 or Cy3) on the array, thereby accounting for the specific activity of the probes. That is, all Cy5 spots were normalized to the average Cy5 signal and vice versa for Cy3. Signals that were higher under control conditions were inverted to permit direct comparison between induction and repression ratios. Correlation among reverse-labeled repeat hybridizations with forward-labeled samples ranged from 0.756 to 0.998, and the standard deviation of duplicate (but reverse-labeled) induction ratios ranged from 0.08 to 0.45, providing a measure of reproducibility.

RESULTS

Generation of AI-2 signaling differential. Our overall objective was to identify all of the *E. coli* ORFs that exhibit a significant increase or decrease (more than twice the standard deviation [SD] of the mean induction ratio) in mRNA abundance caused by a differential in AI-2 signaling activity. MDAI2 (*luxS*) cells, which were unable to produce AI-2 as confirmed using a *V. harveyi* AI-2 activity assay (41), were grown to an OD$_{600}$ of 1.0 (ca. 3 to 4 h), split evenly, and resuspended in either CM exhibiting high AI-2 activity (+AI-2) or identically generated CM deficient in AI-2 signaling activity (−AI-2) (see Materials and Methods). This procedure resulted in a greater than 300-fold difference in AI-2 signaling activity experienced by experimental cultures compared to that for negative controls (Table 1). Comparative measurements of transcript abundance were made by extracting RNA from cells immediately following 20 min of exposure to CM (+AI-2) or identically generated CM (−AI-2). The final cell densities (at 20 min) of experimental and control cultures were identical (OD$_{600}$ of ca. 1.2; 17% change), suggesting that transcriptional changes were not substantially influenced by growth rate or cell density differences of control and experimental samples. Lastly, we performed growth stim-

TABLE 2. Cell division, DNA processing, and morphological genes responding to AI-2

Gene	Gene product and/or function[a]	Induction ratio (fold)
bolA	Possible regulator of murein genes	+10.2
tra8_3	IS30 transposase	+4.4
himA	Integration host factor, alpha subunit; site-specific recombination	+3.9
mreD	Rod-shape-determining protein	+3.6
ompA	Outer membrane protein 3a	+3.2
pin	Inversion of adjacent DNA; at locus of e14 element	+3.2
holE	DNA polymerase III, theta subunit	+3.1
nohB	Bacteriophage DNA packaging protein	+3.0
dnaQ	DNA polymerase III, epsilon subunit	+2.8
csrA	Carbon storage regulator; controls glycogen synthesis, gluconeogenesis, cell size and surface properties	+2.8
mrdB	Rod-shape-determining membrane protein; sensitivity to radiation and drugs	+2.6
b2505	Putative outer membrane lipoprotein	−2.7
cspC	Cold shock protein, transcription antiterminator	−2.7
lpp	Murein lipoprotein	−2.7
yfjN	Putative cell division protein	−2.7
cspI	Cold shock-like protein	−2.8
osmB	Osmotically inducible lipoprotein	−2.9
ftsE	ATP-binding component of a membrane-associated complex involved in cell division	−3.1
cspE	Cold shock protein, chromatin partitioning, and nucleoid structure	−3.1
dicB	Inhibition of cell division	−3.2
murD	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	−3.4
gef	Gef protein interferes with membrane function when in excess, cell killing	−6.6

[a] Genome information is from Blattner et al. (9).

ulatory assays (see Materials and Methods) to determine whether the culture growth rates were affected by the presence of AI-2. The results (not shown) confirmed that there was no significant difference in growth rate between cultures exposed to CM (+AI-2) or CM (−AI-2). Thus, it was concluded that under the conditions tested here, AI-2 does not stimulate or inhibit cell growth.

Identification of quorum-regulated genes in *E. coli*. Using DNA microarrays, we identified 242 genes representing approximately 5.6% of the entire genome that were upregulated (154 total genes) or repressed (88 total genes) more than 2.3-fold (corresponding to 2 SD above the mean induction ratio over the entire array) in the presence of AI-2. A total of 139 genes changed more than 2.9-fold (3 SD), and 23 genes changed more than 5-fold, including *frwC* (33.0-fold), *yeiK* (25.4-fold), and *yidS* (21.3-fold). On the contrary, 25 genes were more than 5-fold repressed, notably *b2650* (27.8-fold), *thiH* (19.2-fold), and *b2247* (15.2-fold). The entire dataset of induction ratios for all 4,290 annotated *E. coli* ORFs can be accessed at http://www.umbi.umd.edu/~cab/bentley/AI-2_array.html.

Involvement of AI-2 in multiple physiological processes. Importantly, we observed that a large number of the responding genes comprised three broad functional categories, according to Riley and Labedan (35). To be specific, we identified 22 genes involved in cell division, DNA processing, and morphological (cell shape) processes (Table 2), which was consistent with previous findings that a quorum sensing mechanism regulates DNA replication and cell division (48, 52). Twenty-three genes were involved in processes known to be quorum-regulated in other gram-negative bacteria, such as virulence, biofilm and exopolysaccharide formation, cell motility, and other surface-associated phenomena (Table 3) (14, 21, 31, 32, 45). Lastly, a cluster of 28 genes involved in small-molecule metabolism included genes which, to date, have not been implicated

in cell-cell communication but may provide a link between central metabolism and quorum signaling, perhaps for the production and degradation of AI-2 itself (Table 4). Interestingly, addition of AI-2 did not significantly affect any of the currently known quorum sensing genes in *E. coli* under the timing and conditions studied here (Table 5) but did significantly alter expression of 10 putative signal transduction-associated genes (Table 6). Also, a large percentage (ca. 60%) of the genes exhibiting 2.9-fold changes (3 SD) were putative ORFs with no currently known function (Table 7).

Interestingly, a putative σ^{54}-dependent transcriptional activator, *ygeV*, whose gene product contained a putative σ^{54} interaction domain as well as a DNA-binding helix-turn-helix (HTH) motif, exhibited significant homology (61% identity) to the LuxO quorum response regulator of *V. harveyi* (28) and was upregulated 3.6-fold. Alignment of the central portions of LuxO and YgeV as well as several other AI-2 responding proteins is depicted in Fig. 1. In addition, *yhbH*, encoding a putative σ^{54} modulating protein and having 85% identity to ORF95 of *V. harveyi* (Fig. 1), was observed to increase 2.5-fold (Table 6). Interestingly, *rpoN* σ^{54} levels were relatively unchanged (decreased 1.08-fold), suggesting that corresponding σ^{54} levels are either unchanged in response to AI-2 or regulated at the translational level, perhaps by YbhH-mediated modulation. Of note, the genetic organization of the *rpoN* chromosomal region of *E. coli* (Fig. 2) (24) is almost identical to that of the *V. harveyi rpoN* region, as will be discussed later. Finally, we found that expression of *rbsB*, encoding a ribose binding protein homologous to LuxP (Fig. 1) and thought to bind AI-2 directly (28), was relatively unchanged (1.4-fold). To determine whether ribose had a direct effect on AI-2 quorum sensing in *E. coli*, W3110 cells harboring a *ftsQA* p2 promoter probe plasmid that is positively regulated by SdiA (39) and responds to AI-2 (15) were exposed to varying concentrations of ribose. Plasmid-bearing cells were grown in LB medium plus

TABLE 3. Genes comprising quorum-regulated processes (i.e., virulence, biofilm formation, motility, surface, and outer membrane-associated functions)

Gene or B number	Gene product and/or function[a]	Induction ratio (fold)
hha	Hemolysin expression-modulating protein	+11.1
wzb	Probable protein-tyrosine-phosphatase	+6.2
ompG	Outer membrane porin protein	+5.1
smpA	Small membrane protein A	+4.5
yadK	Putative fimbrial protein	+3.8
rcsB	Positive response regulator for colanic capsule biosynthesis (sensor, RcsC)	+3.5
yadN	Putative fimbrial-like protein	+3.5
crl	Transcriptional regulator of cryptic *csgA* gene for curli surface fibers	+3.5
rfaJ	UDP-D-glucose:(galactosyl)lipopolysaccharide glucosyltransferase	+3.4
rnk	Regulator of nucleoside diphosphate kinase	+3.1
motB	Enables flagellar motor rotation, linking torque machinery to cell wall	+3.1
b1502	Putative adhesin; similar to FimH protein	+3.0
tap	Methyl-accepting chemotaxis protein IV, peptide sensor receptor	+2.9
pstC	High-affinity phosphate-specific transport system, cytoplasmic membrane component	+2.8
yehA	Putative type 1 fimbrial protein	+2.7
rfaY	Lipopolysaccharide core biosynthesis	+2.5
rfaD	ADP-L-glycero-D-mannoheptose-6-epimerase	−2.7
uhpT	Hexose phosphate transport protein	−2.7
cheW	Positive regulator of CheA protein activity	−2.7
b1629	Putative membrane protein	−2.8
evgS	Putative sensor for regulator EvgA and homologous to *B. pertussis bvgS*	−2.8
fliP	Flagellar biosynthesis	−2.9
nlpC	Lipoprotein	−2.9
flgN	Protein of flagellar biosynthesis	−3.7

[a] Genome information is from Blattner et al. (9). Bold-faced genes exist within known operons

50 mM glucose, and experimental cultures were supplemented with 2 g of either L- or D-ribose per liter. Results demonstrated that L-ribose moderately stimulated expression of the quorum-regulated *ftsQA* genes through the p2 promoter relative to controls containing no L-ribose (Fig. 3), while exposure to D-ribose resulted in nearly identical *ftsQA* induction levels as negative controls (data not shown). Interestingly, we found that addition of L-ribose at similar concentrations (ca. 1 to 2 g/liter) resulted in moderate induction of the *lux* genes in the *V. harveyi* AI-2 reporter assay (ca. 300- to 400-fold activation;

TABLE 4. Small molecule metabolism induced by AI-2 signaling

Gene	Gene product and/or function[a]	Induction ratio (fold)
frwC	PTS system, fructose-like enzyme II component	+33.0
fpr	Ferredoxin-NADP reductase	+10.9
ntpA	dATP pyrophosphohydrolase	+4.5
bioH	Biotin biosynthesis; reaction prior to pimeloyl Coenzyme A	+3.7
lysP	Lysine-specific permease	+3.6
edd	6-Phosphogluconate dehydratase	+3.3
trpR	Regulator for trp operon and aroH; trp aporepressor	+3.2
gpmB	Phosphoglyceromutase 2	+3.2
marB	Multiple antibiotic resistance protein	+3.1
psiF	Induced by phosphate starvation	+2.9
dgt	Deoxyguanosine triphosphate triphosphohydrolase	+2.8
glcB	Malate synthase G	+2.8
caiF	Transcriptional regulator of cai operon	+2.8
tdcA	Transcriptional activator of tdc operon	+2.7
tsx	Nucleoside channel; receptor of phage T6 and colicin K	+2.6
rfbC	dTDP-6-deoxy-D-glucose-3,5 epimerase	−2.7
potB	Spermidine/putrescine transport system permease	−3.1
wcaH	GDP-mannose mannosyl hydrolase	−3.1
usg	Putative PTS system enzyme II A component	−3.3
pheL	Leader peptide of chorismate mutase-P-prephenate dehydratase	−3.5
carB	Carbamoyl-phosphate synthase large subunit	−3.7
potA	ATP-binding component of spermidine/putrescine transport	−5.1
mtlR	Repressor for mtl	−5.1
ilvL	*ilvGEDA* operon leader peptide	−5.4
hyfA	Hydrogenase 4 Fe-S subunit	−5.6
ugpC	ATP-binding component of sn-glycerol 3-phosphate transport system	−6.2
thiH	Thiamin biosynthesis, thiazole moiety	−19.2

[a] Genome information is from Blattner et al. (9). Bold-faced genes exist within a known operon.

TABLE 5. Response of known *E. coli* quorum sensing genes to AI-2

Quorum-regulated gene	Function or description	Fold increase	Reference
ftsQ	Cell division	1.1	48
ftsA	Cell division	1.0	48
ftsZ	Cell division	1.4	48
sdiA	Positive regulator of cell division	2.0	39
rpoS	Stationary phase sigma factor	1.2	39
cysK	O-acetylserine lyase A	1.5	3
astD	Putative succinylglutamate-semialdehyde dehyrogenase	1.3	3
tnaB	Glutamate:succinate semialdehyde aminotransferase	1.1	3
gabT	Low-affinity tryptophan permease	1.4	3

data not shown), suggesting L-ribose might act as an analogue or precursor that can trigger the AI-2-stimulated quorum response.

Independent measure of RNA levels confirms AI-2 stimulatory effect on gene expression. Previously, quantitative reverse transcription-PCR (50) and *lacZ* transcriptional fusions (45) were used to independently validate microarray expression data. In a similar manner, total RNA dot blotting was performed as previously outlined (15) and used to quantitatively verify the expression level changes of representative genes identified by microarray analysis. For these confirmatory experiments, RNA samples were harvested from a replicate experiment (see Materials and Methods), and induction ratios were compared between the two methodologies (Table 8). Reassuringly, the expression changes of 8 genes (out of 9 tested) were confirmed using total RNA dot blotting, which represented agreement (ca. 89%) similar to that previously reported (50). The lone discrepancy, *thiH*, was significantly repressed (19.2-fold) according to comprehensive transcript profiling but was upregulated (2.8-fold) according to the dot blotting procedure. The source of this difference, while not definitively resolved, was possibly due to high background signal in the film-based development associated with RNA blotting.

TABLE 6. Putative signal transduction-associated genes that significantly respond to the AI-2 quorum signal

Gene or B number	Probable gene product[a]	Induction ratio (fold)
ylcA	Putative two-component transcriptional regulator	+4.4
b2380	Putative sensor protein	+3.9
ygeV	Putative σ^{54}-dependent transcriptional regulator	+3.6
b2248	Putative regulator	+3.6
ybiF	Putative transmembrane subunit	+3.4
yqhC	Putative AraC-type regulatory protein	+2.7
yhhM	Putative receptor	+2.6
yhbH	Probable σ^{54} modulation protein	+2.5
ycgE	Putative transcriptional regulator	−2.6
ycjZ	Putative transcriptional regulator, LysR type	−3.3

[a] Genome information is from Blattner et al. (9).

TABLE 7. Genes of unknown class or function significantly induced or repressed by AI-2 quorum signaling

| Gene or B number	Induction ratio (fold)
yeiK	+25.4
yidS	+21.3
pqqL	+12.7
yghB	+11.3
ycbR	+9.8
yaeT	+9.8
b1506	+9.7
ydfE	+9.7
yggE	+9.6
b0100	+7.8
b2145	+7.0
ybcJ	+6.5
b3254	+6.2
yccE	+5.4
ydaC	+4.9
yaiV	+4.8
ybiM	+4.7
yddM	+4.5
b1543	+4.3
yagH	+4.3
b2432	+4.2
b2875	+4.2
yhbP	+4.1
ydfA	+4.1
b2326	+4.0
b2792	+3.9
yebG	+3.9
yqgB	+3.8
ybhH	+3.7
yhcO	+3.6
b2868	+3.5
yhiE	+3.5
ydiB	+3.4
yibA	+3.3
ygeF	+3.3
b1152	+3.3
yecN	+3.3
yjhC	+3.2
ycbG	+3.2
b0245	+3.1
yfeH	+3.1
b1364	+3.1
b1155	+3.1
b1582	+3.0
b1445	+3.0
yebA	+3.0
yabI	+3.0
b1567	+3.0
b1438	−3.0
b2859	−3.0
ydcD	−3.1
yfiK	−3.1
ybeB	−3.1
ybiK	−3.1
ydjA	−3.2
yqjC	−3.3
b4250	−3.3
yjgX	−3.5
yqjH	−3.6
b1963	−3.7
ycjC	−3.9
yifM	−3.9
b2390	−3.9
b2511	−4.2
b2748	−4.4
b1553	−4.7
ybgF	−5.7
yaiC	−5.9
ygaF	−6.1
yifN	−6.8
b2254	−6.8
b2363	−7.7
yecE	−7.7
ymgC	−8.4
b2247	−15.2
b2650	−27.8

[a] Genome information is from Blattner et al., (9).
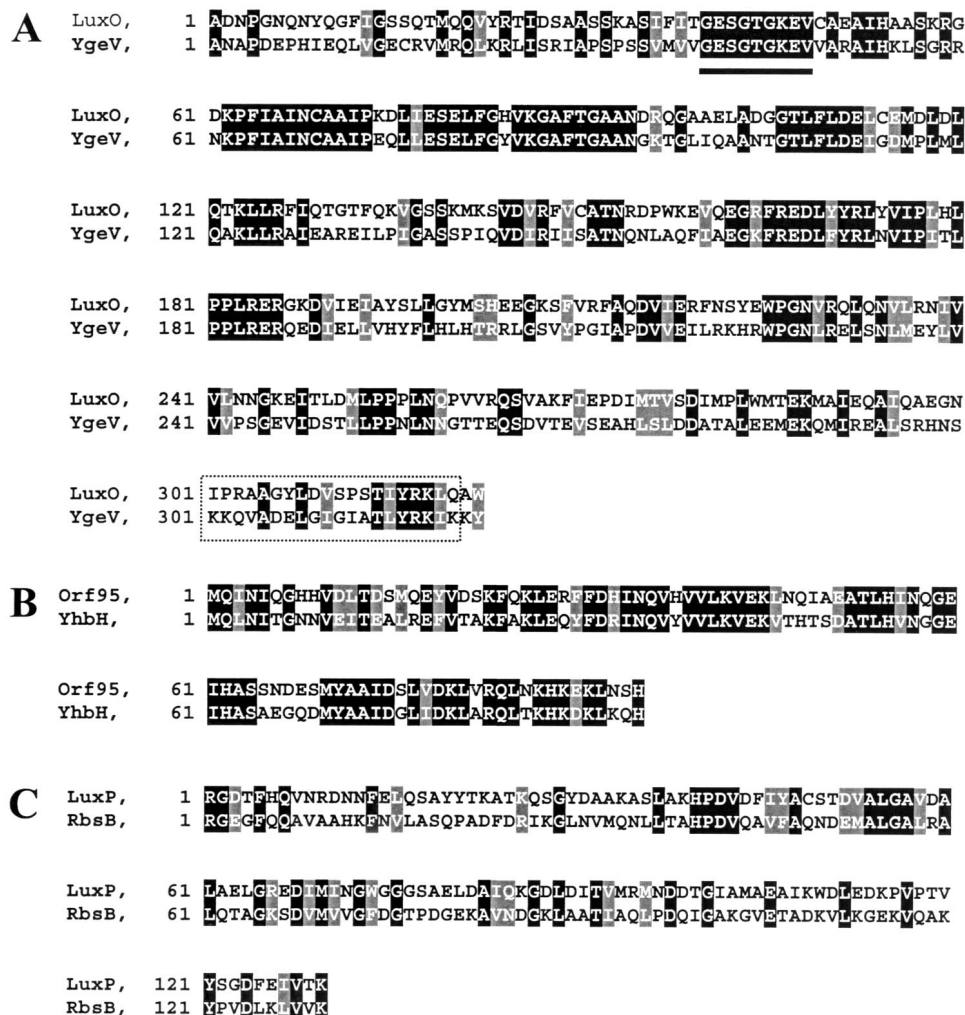
FIG. 1. Alignment of LuxO of *V. harveyi* with a putative σ⁵⁴-dependent transcriptional activator of *E. coli* (A), YgeV ORF95 of *V. harveyi* with YhbH of *E. coli* (B), both putative σ⁵⁴-modulating proteins, and LuxP of *V. harveyi* with the ribose periplasmic binding protein of *E. coli*, RbsB (C). Amino acids that match the consensus generated for the two sequences are boxed in black. The glycine-rich region encoding the nucleotide binding domain common of σ⁵⁴-interacting proteins is underlined, while the putative HTH DNA binding domains for LuxO and YgeV are boxed by a dashed line.

DISCUSSION

To determine the transcriptional response of *E. coli* to the AI-2 quorum signal, cells deficient in AI-2 production (W3110 *luxS*::Tcʳ) (15) were exposed for 20 min to medium conditioned by either AI-2-producing (W3110 *luxS*⁺) or non-AI-2-producing (W3110 *luxS*) cells. Generation of a 300-fold differential in AI-2, confirmed by an AI-2 activity assay (41), led to the discovery that almost 6% of the *E. coli* genome (242 genes) was modulated more than 2.3-fold (2 SD) in response to AI-2-regulated quorum sensing. Consistent with these results, it has been conservatively estimated that 3 to 5% of all genes in *P. aeruginosa* are regulated by acyl-HSL quorum signaling, as was partially demonstrated by screening a library of *lacZ* promoter probes which uncovered 270 genes showing more than twofold stimulation (70 showed more than fivefold stimulation) (51).

It is now well known that *V. harveyi* uses a species nonspecific signaling pathway mediated by AI-2 for regulating *lux* gene expression (7, 8, 41). Recognition of AI-2 by LuxP, homologous to the ribose binding protein of *E. coli*, has been proposed to transmit the signal to the LuxU phosphorelay protein via interaction with a hybrid sensor kinase, LuxQ (7, 8, 28). In turn, the signal is relayed to a central regulator, LuxO,
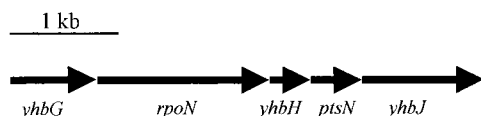

FIG. 2. Genetic organization of the *E. coli rpoN* chromosomal region. The genetic organization of this region is similar to that described for the *rpoN* region of *Vibrio cholerae* and *V. harveyi*. *yhbG* encodes a probable ATP-binding cassette (ABC) transporter protein, *yhbH* encodes a putative σ⁵⁴ regulatory protein much like *orf95* of *V. harveyi*, *ptsN* encodes a PTS system nitrogen regulator, and *yhbJ* has no known function (24).
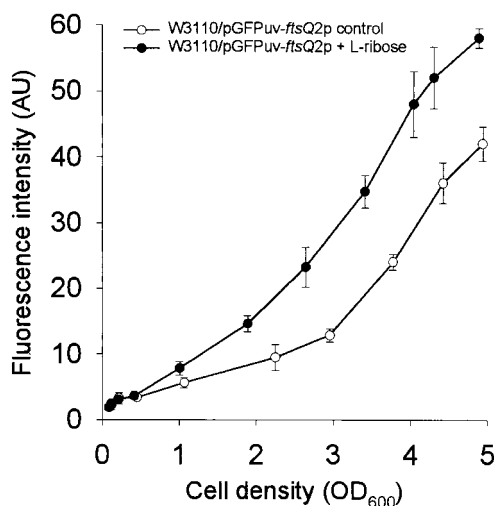
FIG. 3. Induction of *ftsQA* expression through the p2 promoter by W3110/pGFPuv-*ftsQ*2p reporter cells in LB medium plus 50 mM glucose or LB medium plus 50 mM glucose and supplemented with L-ribose (2 g/liter). AU, arbitrary units.

which, upon interaction with σ^{54}, indirectly represses the *lux* operon (28). In this study it was observed that AI-2 induced a gene encoding a σ^{54}-dependent transcriptional activator (*ygeV*) as well as a σ^{54} modulator (*yhbH*), leading to our postulation that *E. coli* may employ σ^{54} during quorum sensing in a fashion analogous to that of *V. harveyi*. The striking similarity of the *rpoN* chromosomal regions of *V. harveyi* and *E. coli* along with the observed increase in *yhbH* expression (2.5-fold) suggests similar regulatory controls exist. Finally, in addition to regulating light production, LuxO and σ^{54} regulate siderophore production and colony morphology, demonstrating that multiple processes are regulated by quorum sensing in *V. harveyi* (28). In the present study, quorum-regulated genes existed in several functional classes, and while systematic determination of quorum-controlled processes still remains, it is clear that

TABLE 8. Fold induction of transcripts in response to AI-2 quorum signal as determined by microarray probing and RNA dot blotting

| Gene | Fold change with[a]: | |
	RNA dot blotting	Microarray probing
ftsQ	1.0	1.1
sdiA	1.0	2.0
ompA	4.7	3.2
rpoS	1.3	1.2
rcsB	4.3	3.5
thrS	4.5	3.1
ygeV	3.9	3.6
thrS	27.2	33.0
thiH	2.75	19.2

[a] RNA dot blotting (and microarray) results were obtained as outlined in Materials and Methods. Reported values are intensity obtained from experimental RNA samples divided by intensity obtained from control RNA and are the averages of three replicate samples. Microarray and blotting RNA samples were from replicate experiments.

AI-2 also affects multiple processes in *E. coli* and perhaps enables population-wide coordination of these events.

Determination of quorum-regulated processes in *E. coli* has been elusive, as discovery of a quorum signal was only recently made. However, the finding that an extracellular factor exhibited inhibitory activity during initiation of DNA replication (52) provided preliminary evidence that *E. coli* employed a quorum sensing mechanism. Consistent with this observation, a gene encoding the integration host factor alpha subunit (*himA*) involved in the replication of the *E. coli* chromosome, and *holE*, a gene encoding the θ subunit of DNA polymerase III (necessary for elongation), were upregulated 3.9- and 3.1-fold, respectively, in response to AI-2. A role for quorum sensing in cell division was first demonstrated by SdiA-mediated changes in *ftsQAZ* expression from the p2 upstream promoter induced by CM (20, 39, 48) and later attributed to AI-2 (15). In this study, expression of *sdiA*, a *luxR*-type transcriptional regulator, was observed to increase only slightly (2.0-fold) in response to AI-2, indicating either that AI-2 does not significantly affect *sdiA* expression or that the effect occurs on a different time scale than that tested here. Accordingly, expression of *ftsQ*, *ftsA*, and *ftsZ* were relatively unchanged (1.1-fold, 1.0-fold, and 1.4-fold, respectively), although *ftsE*, encoding an ATP-binding component of a membrane-associated complex involved in cell division, decreased 3.1-fold. While the results presented here appear to contradict the earlier findings of increased AI-2-mediated *ftsQAZ* transcription through p2, we conclude that the p2 construct alone behaves differently than the p1 and p2 promoters acting in concert. This observation is supported by experiments using CM that showed that an extracellular factor stimulated *ftsQA* expression fivefold from P2$_{ftsQ}$ but only two- to threefold from both P1$_{ftsQ}$ and P2$_{ftsQ}$ together (39). Bassler and colleagues also reported that fusions of both p1 and p2 promoters to *lacZ* were not significantly altered by the presence of AI-2. Therefore, that we earlier observed *ftsQAZ* expression changes stimulated by AI-2 through promoter p2 alone suggests that under physiological conditions *ftsQAZ* expression is influenced by overlapping regulation from the neighboring *rpoS*-dependent P1$_{ftsQ}$ promoter. In fact, it has been documented that the two *ftsQA* promoters are regulated differentially, with expression from P2$_{ftsQ}$ occurring throughout growth and dependent on *sdiA*, while that from P1$_{ftsQ}$ (a gearbox promoter) increases as the growth rate declines and is dependent on *rpoS* (1, 39, 48).

Additional evidence that quorum sensing positively regulates cell division was the observed 3.2-fold decrease in expression of *dicB*, an inhibitor of the synthesis and activity of FtsZ. While *sdiA* is known to positively regulate cell division, inhibition of division can occur via derepression of *dicB*, whose gene product cooperates with MinC to inhibit FtsZ assembly, blocking septation at all potential division sites (13). Therefore, repression of *dicB* by the AI-2 quorum signal might exert additional positive control over cell division. Further, *rcsB*, another *luxR*-type transcriptional regulator protein known to affect colanic acid capsular polysaccharide synthesis, demonstrated a 3.5-fold increase in transcription. The role of *rcsB* in activating the *ftsA* and *ftsZ* genes (10), perhaps through P1$_{ftsQ}$ or P2$_{ftsQ}$, coupled with its increased transcription induced by AI-2, suggests that a quorum regulatory mechanism governs these distinct processes. In further support of a role for AI-2 in

exopolysaccharide biosynthesis was the increased transcription of *wzb* (6.2-fold increase), a gene found within the colanic acid gene cluster and, in conjunction with *wzc*, that is known to participate in the export of the extracellular polysaccharide colanic acid from the cell to the medium (46).

Using RegulonDB software (36) available at http://www.cifn .unam.mx/regulondb/, we obtained a predicted 81-bp promoter of *rcsB*, which was subsequently input to GRASP-DNA software (38) available at http://www-bioeng.ucsd.edu/~grasp /home.html and was used to identify homologous putative DNA-protein binding sites. This analysis revealed significant homologous regulatory regions upstream of *rcsB* and *ompG*, which was interesting, as *ompG* expression was similarly upregulated (5.1-fold). Additionally, the threonyl-tRNA synthetase, *thrS*, having ca. 46% identity to a short segment of *luxU* (data not shown), was observed to increase 3.1-fold. A putative 81-bp promoter of *thrS* had upstream promoter homology with *rfaJ*, a lipopolysaccharide biosynthesis gene which was similarly upregulated (3.7-fold). Of note, *thrS* mRNA has been shown to accumulate with increasing growth rate (11), which might be a consequence of its response to AI-2, which also accumulates in a growth-rate-dependent fashion (15). Similarly, operon structure could be probed using RegulonDB. All of the AI-2-responding genes were examined using the RegulonDB graphical interface, and while many of the genes occurred within predicted (as opposed to known) operons, only the *cheAW-motAB*, *potABCD*, and *rfaQGPSBIJYZK* operons contained multiple genes responding to AI-2. In the first two cases, two genes responded similarly (e.g., *rfaY* and *rfaJ*, and *potA* and *potB*), while in the last case the responses of *cheW* and *motB* were in different directions. In all cases putative promoters are located between the identified genes, so a differential response might be expected. Interestingly, it has been shown that mutation of the *att* operon of *Agrobacterium tumefaciens*, a 10-kb region of 9 ORFs bearing strong homology to the *pot* operon of gram-negative bacteria, resulted in avirulence and inability to attach to plant cells (31). However, the ability of *att* mutants to bind to host cells was restored by the addition of conditioned medium during incubation of the bacteria with the host, suggesting that either efflux or uptake of an extracellular factor necessary for attachment through the spermidine pathway was blocked in mutant strains. This is very interesting in light of the 3.1- and 5.1-fold decrease in *potAB* expression in response to *E. coli* CM containing AI-2. This approach demonstrates that combination of global expression data with powerful bioinformatic algorithms, such as GRASP-DNA and RegulonDB, can elucidate potential regulatory overlap from transcriptional data.

Transcription of several other exopolysaccharide (*rcsB*, *rfaD*, *rfaJ*, *rfaY*, and *rnk*)- and virulence (*hha* and *evgS*)-related genes responded to AI-2. Outer surface polysaccharides are important components in the virulence of many pathogens, as they mediate direct interaction between bacteria and their immediate environment. While the *E. coli* strain studied here was not virulent, this was not entirely surprising, as many pathogenic gram-negative bacteria regulate virulence via quorum sensing (14). For example, *hha*, encoding the regulator of the hemolysin operon and reported to mediate the environmental regulation of virulence factors in *P. aeruginosa* (51), increased 11.1-fold in response to AI-2. Interestingly, *ompA* expression

increased 3.2-fold, consistent with existing evidence that OmpA, in addition to maintaining outer membrane integrity, might play an important role in virulence of *Pasteurella haemolytica* (30). Of note, *ompA* expression was reported to decrease by 59% in an *E. coli hha* mutant (5). Also, the putative *E. coli* virulence gene, *evgS*, which constitutes a two-component system with the *luxR*-type regulator *evgA* that is structurally and functionally similar to the *bvgAS* two-component regulator of virulence factors in *Bordatella pertussis* (44), was repressed 2.8-fold. Finally, *csrA*, a global repressor of glycogen biosynthesis that alters stability of specific mRNA targets (29), increased 2.8-fold. While *csrA* has not been directly associated with quorum sensing in *E. coli*, structural and functional homologues regulate invasion genes in *S. enterica* serovar Typhimurium (2) and extracellular enzymes and *N*-(3-oxohexanoyl)-L-homoserine lactone quorum signals and pathogenicity in *Erwinia carotovora* (*rsmA*) (12). Additionally, *csrA* has been documented to affect cell size and surface properties, which is in agreement with the transcriptional changes of several murein sacculus-associated morphological genes. These genes include *bolA* (10.2-fold), an *ftsZ*-dependent regulator of the murein genes (1), and *mreD* (3.6-fold), encoding a rod-shape-determining protein as well as several exoskeletal (fimbriae, flagella, and curli surface fibers) genes, such as *yadK* (3.8-fold), *yadN* (3.5-fold), *crl* (3.5-fold), *b1502* (3.0-fold), *yehA* (2.7-fold), *fliP* (−2.7-fold), and *flgN* (−3.7-fold). The coupling of morphological gene expression to AI-2 quorum signaling might ensure that the cytoskeletal framework be temporally regulated in association with growth phase and cell cycle progression.

Overall, our results yield significant insight into possible AI-2-coordinated changes in gene regulation that might temporally and spatially unify processes such as cell division, morphogenesis, and cell surface architecture. Interestingly, as many as 10 known sensors and/or transcriptional regulators as well as 10 putative signal transduction genes responded to increased AI-2 signaling, which, along with several other candidate genes and processes, warrant further study in the context of AI-2-stimulated quorum regulation. It is clear that quorum sensing is a complex signaling circuit that is built upon transducing elements that allow integration and channeling of multiple environmental cues, and elucidation of AI-2-controlled genes is a critical first step in mapping the metabolic pathways that define the *E. coli* quorum circuit.

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